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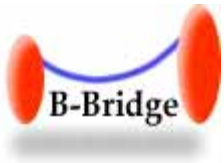

esRAGE ELISA Kit
User Manual

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See List of Components for Storage Conditions
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I. Introduction and Protocol Overview

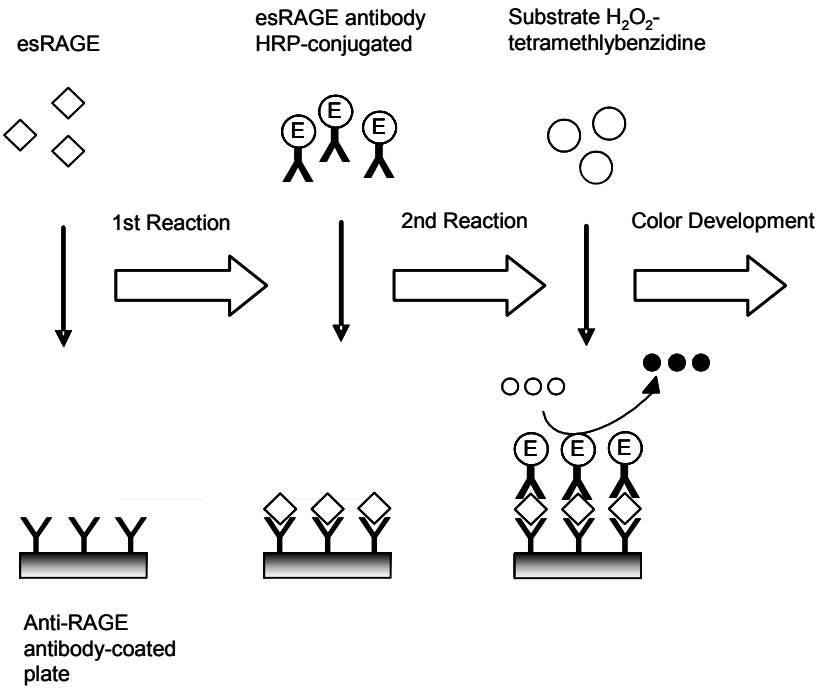
In multicellular organisms, most cellular and plasma proteins undergo a nonenzymatic glycation by the covalent binding of reduced sugars to the amino groups on proteins. A balance exists between early glycation products and non-glycated proteins during homeostasis. Under the condition of prolonged hyperglycemia as in diabetes, these early stage glycation products undergo additional chemical rearrangements to form irreversibly bound advanced glycation end products (AGE) [1, 2]. AGE has been shown to be a major factor in vascular cell derangement in diabetic patients[3, 4]. AGE has also been implicated in Alzheimer's Disease by the glycation of amyloid-β peptide and microtubule-associated protein tau[5]. The receptor for AGE (RAGE), a 45kDa protein and a member of the immunoglobulin superfamily of cell surface molecules, is found in mononuclear phagocytes, endothelium, vascular smooth muscle, and the central nervous system[6, 7, 8]. RAGE is a multi-ligand receptor. The most pathological consequence of the AGE-RAGE engagement is induction of oxidative stress, activation of nuclear factor kB (NF-kB), resulting in disruption of homoeostatic functions in the vasculature[9, 10, 11]. AGE has also been shown to up-regulate the RAGE expression in endothelial cells[12].

Translation of RAGE mRNA isolated from human endothelial cells revealed a novel splice variant named endogenous secretory receptor (esRAGE) which can capture AGE and neutralize the effects of AGE on cells[3, 13]. Endogenous secretory RAGE has been found circulating in blood and in extracellular fluids of our bodies[13].

The B-Bridge **esRAGE ELISA Kit** is designed to measure the concentration of human esRAGE in serum, plasma, and tissue culture medium.

The principle of the assay is shown in Figure 1. Standards or samples and detection antibody (esRAGE antibody horseradish peroxidase conjugated) are incubated in an anti-RAGE antibody coated 96-well microtiter plate. After 16-24 hour incubation and four washing, substrate is added to each well. The plate is incubated for 30 minutes. The enzymatic reaction is stopped by the addition of stop solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of esRAGE. A standard curve is constructed by plotting absorbance values versus esRAGE concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

Figure 1. Assay Principle



II. List of Components

- Store all components at 2-8ºC. DO NOT FREEZE.

1	**PRIMARY ANTIBODY-COATED PLATE** One 96-well plate with adsorbed anti-RAGE antibody Plate is provided in a resealable foil pouch with desiccant.	1 Plate
2	**DETECTION ANTIBODY** esRAGE antibody-horseradish peroxidase (HRP) conjugated	1 Bottle (11 ml)
3	**SUBSTRATE** 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide	1 Bottle (15 ml)
4	**STOP SOLUTION** 1 M sulfuric acid	1 Bottle (15 ml)
5	**BUFFER** Phosphate buffered saline containing bovine serum albumin	1 Bottle (5 ml)
6	**esRAGE STANDARD** Human esRAGE, 1.6 ng	1 Bottle (lyophilized)
7	**5X WASH SOLUTION** Phosphate buffered saline with Tween 20	2 Bottle (50 ml each)

MSDS forms are available on our website—please visit www.b-bridge.com

III. Additional Materials Required

The following materials are required, but not supplied:

- Graduated cylinder
- Micropipette(s) and disposable pipette tips
- Null strips for 96-well plate
- 96-well plate or manual strip washer
- Paper towels or absorbent paper
- Plate reader capable of measuring absorbance at a wavelength of 450 nm (reference filter at 650 nm, optional)
- Well-closed containers such as microtubes (1.5 ml or more in capacity)
- Refrigerator, 2-8ºC

IV. Reagent Preparation and Storage

Allow all the reagents to come to room temperature (20-30°C) prior to the start of reagent preparation.

1. 1X Wash Solution
   If crystals are observed in the 5X Wash Solution (⑦ bottle), warm the bottle in a 37°C water bath until the crystals disappear. Prepare 1X Wash Solution by mixing 50 ml of the 5X Wash Solution with 200 ml of deionized water or equivalent. Mix well. After preparation, store 1X Wash Solution at 2-8°C. The 1X Wash Solution is stable for 1 month at 2-8°C.

2. esRAGE Standard
   Reconstitute esRAGE standard (⑥ bottle) with 0.5 ml of distilled water. Prepare each esRAGE assay standard (1.6 ng/ml, 0.8 ng/ml, 0.4 ng/ml, 0.2 ng/ml, 0.1 ng/ml, 0.05 ng/ml) by serially diluting the reconstituted standard with buffer (⑤ bottle). Use the undiluted esRAGE Standard (3.2 ng/ml) and buffer for the 3.2 ng/ml and 0 ng/ml assay standards, respectively.

   Assay standards should be prepared prior to use and any unused assay standards should be discarded.

   Reconstituted esRAGE standard is stable for 1 month at 2-8°C.

3. Other kit reagents
   Anti-RAGE antibody-coated plate, esRAGE antibody horseradish peroxidase (HRP)-conjugated, substrate, stop solution, and buffer are ready for use. Once opened, these reagents are stable for 3 months at 2-8°C.

Note: The unopened reagents, if properly maintained at 2-8°C, are stable until the expiration date printed on the box label.

Do not mix reagents from different kits unless they have the same lot number.

V. Sample Preparation

Samples may be diluted with ⑤ buffer, if quantitation of the sample protein is above the 3.2 ng/ml data point on the standard curve.

VI. Human esRAGE ELISA Protocol

Note: Allow all reagents to come to room temperature (20-30°C) prior to the start of the assay and prepare 1X Wash Solution and esRAGE Standards as described in the previous sections.

1. Remove Primary Antibody-Coated Plate from its foil pouch. Remove any unneeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 2-8°C. If a 96-well plate washer is used, the plate frame should be completely filled with wells by adding as many null strips as necessary. Identify well position(s) for each sample on a data sheet or plate map.

2. Add 100 µl of esRAGE antibody HRP-conjugate to the appropriate number of antibody-coated wells.

3. Add 20 µl of assay standards (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 ng/ml) or sample to the plate and mix well by pipetting. All assay standards and samples should be run in duplicates. Every plate must include the assay standards to properly correlate the sample readings.

4. Cover plate(s) securely with a plate sealer and incubate at 4-10°C for 16-24 hours. The plates should not be agitated or rocked during the incubation period.

5. Aspirate the reaction mixture from each well.

6. Wash each well with 350 µl of 1x wash solution.

7. Aspirate the wash solution

8. Repeat steps 6 and 7 three times for a total of 4 washes

9. Invert the plate and gently tap on a clean absorbent towel to remove residual droplets of wash solution.

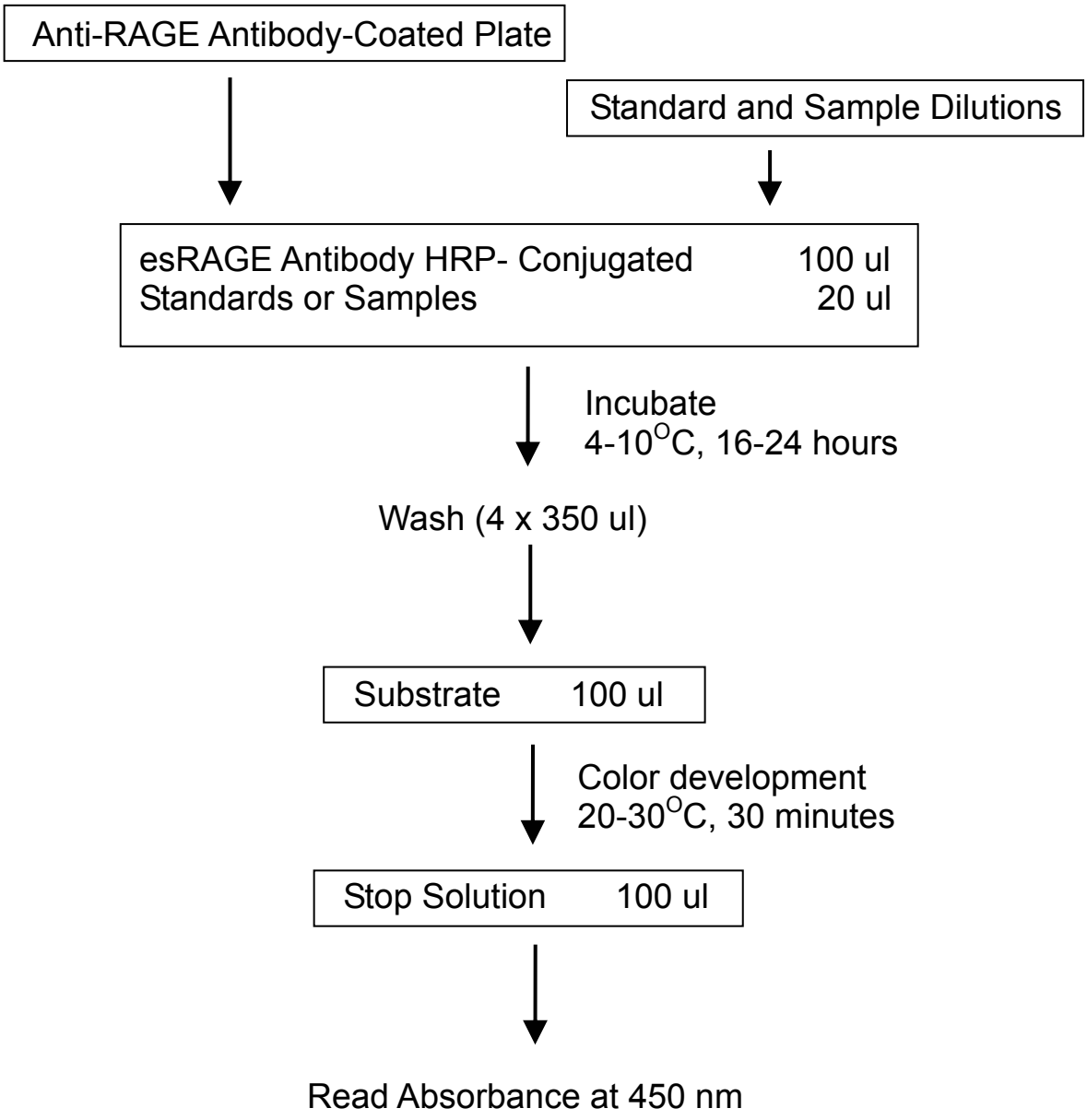
10. Dispense 100 µl of substrate into each well.

11. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with aluminum foil is recommended. Incubate at 20-30°C for 30 minutes.

12. Add 100 µl of stop solution to each well.

13. Read the plate within 30 minutes after stopping the reaction. The plates are measured at wavelength 450 nm. The recommended reference wavelength is 630 nm.

Figure 2. Flow Chart of Assay



VII. Calculation of Results

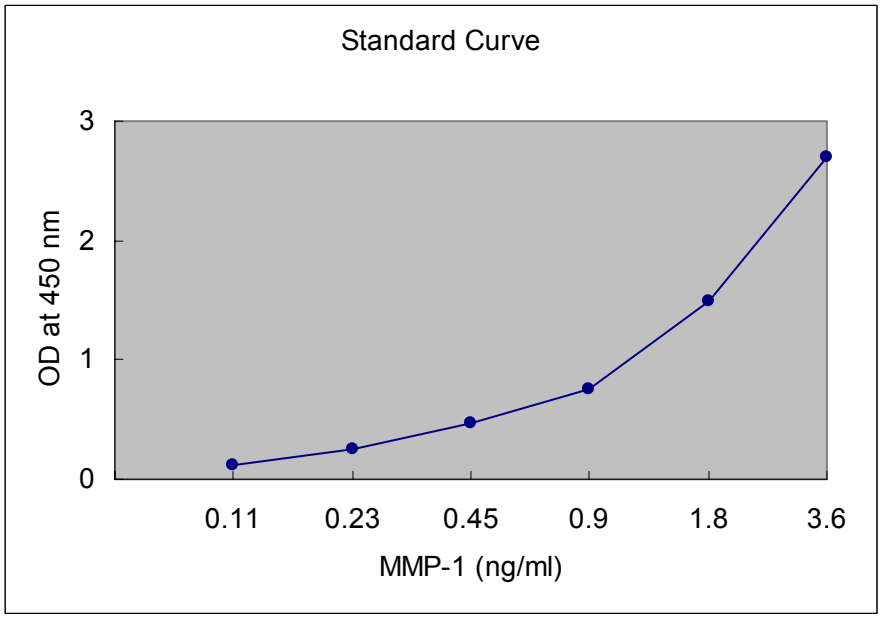
1. Subtract the mean absorbance value of the 0 ng/ml blank from each mean absorbance value of the standard series and samples tested (Net Absorbance).

2. Plot the log of known concentrations of each standard and the calculated Net Absorbance on the X-axis and Y-axis, respectively. Fit an appropriate regression curve to the plotted points.

3. Determine the esRAGE concentrations of the samples by interpolation of the regression curve formula.

4. If samples were diluted, then concentration calculations must be multiplied by the appropriate dilution factor to obtain the correct results for the undiluted samples.

Figure 3. Typical Standard Curve



VIII. Troubleshooting Guide

Troubleshooting Guide

1. Lack of signal or weak signal in all wells
Possible explanations:

- Omission of a reagent or a step.
- Improper preparation or storage of a reagent.
- Assay performed before reagents were allowed to come to 20-30°C.
- Plate reader did not perform well.

2. High signal and background in all wells
Possible explanations:

- Improper or inadequate washing; be certain that all wash volumes and repetitions were correct.
- Improper dilution of detection antibody.
- Overdeveloping; decrease the incubation time before the Stop Solution is added.

3. High background in sample wells only
Possible explanations:

- Sample concentration was too high.
- Improper dilution of detection antibody.

4. Weak signal in sample wells only
Possible explanations:

- Sample concentration was too low.
- Improper dilution of detection antibody.

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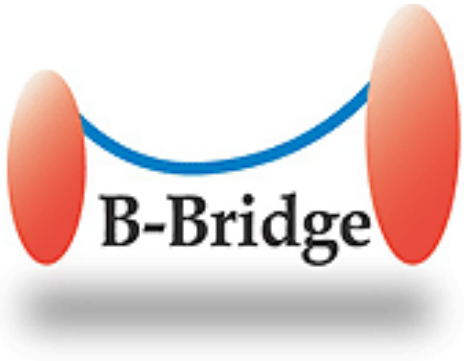

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